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ORIGINAL RESEARCH PAPER

Property control of sophorolipids: influence of fatty acid substrate and blending

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Abstract Sophorolipids (SLs) were synthesized by fed-batch fermentation of *Candida bombicola* on glucose and either palmitic acid (SL-p), stearic acid (SL-s), oleic acid (SL-o) or linoleic acid (SL-l) and the structural distribution accurately determined by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The surfactant properties, including critical micelle concentration (CMC), minimum surface tension (min.ST) and oil-water interfacial tension (IFT) were measured by tensiometry. Minimum STs of 35–36 mN/m were obtained regardless of the substrate while IFTs ranged from 3–5 mN/m with the exception of SL-l, which had an IFT of 7 mN/m. The largest disparity occurred in the CMC values, which ranged from 35 ppm for SL-s to 250 ppm for SL-l. By manually mixing these four SLs in different ratios, it was possible to better control the CMC values without affecting the min.ST or IFT, which will prove beneficial as new applications for SLs are established.

Keywords Critical micelle concentration · Liquid chromatography/mass spectrometry · Minimum surface tension · Sophorolipids · Tensiometry

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Introduction

Biosurfactants are amphiphilic molecules that are produced from biological sources and tend to agglomerate to form micelles or concentrate at interfaces such as air/water, oil/water, or water/solid to reduce the interfacial and/or surface tension of a system. Because of their inherent biodegradability and broad array of functional properties (including emulsification, phase partitioning, wetting, foaming, and surface activity), many of these materials are drawing interest from industry as additives to or substitutes for currently used petrochemical-based products. One class of biosurfactants that is currently receiving industrial consideration is the glycolipids. One of the more common natural glycolipid biosurfactants is sophorolipids (SLs), which are synthesized by a number of yeasts including *Candida bombicola* (most studied system), *Candida apicola* (Hommel and Huse 1993), *Torulopsis magnoliae* (Gorin et al. 1961), and *Rhodotorula bogoriensis* (Nuñez et al. 2004). Sophorolipids are made up of a disaccharide (sophorose; 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranose) linked to a hydroxy fatty acyl moiety by a glycosidic

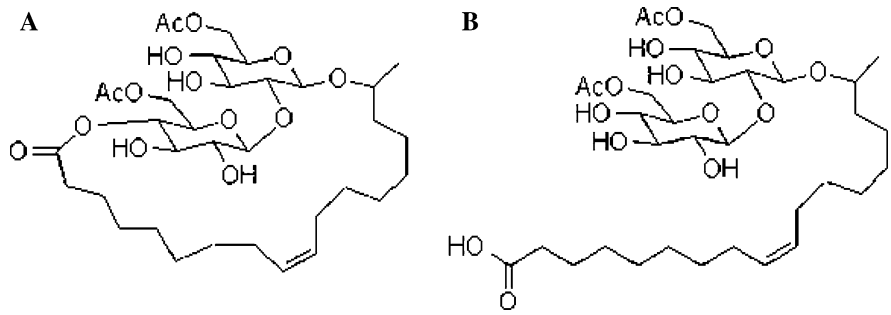


Fig. 1 Structures of 17-L-[2′-*O*-*β*-glucopyranosyl-*β*-D-glucopyranosyl)-oxy]-9-octadecenoic acid 6′,6″-diacetate sophorolipids in the (**a**) 1′,4″-lactone and (**b**) free acid forms

bond between the 1′-hydroxy group of the sophorose sugar and the ω or $\omega - 1$ carbon of the fatty acid (Fig. 1).

Typically, the 6′- and 6″-hydroxy groups of the sophorose are acetylated and the fatty acid chain length varies between 16 and 18 carbons (one exception is the SLs synthesized by *R. bogoriensis*, which include fatty acid side-chains of 22 and 24 carbons; Nuñez et al. 2004); and may be saturated or unsaturated. In addition, the preferred structural conformation of SLs is as a lactone, where the carboxylic acid group of the fatty acid is esterified to the disaccharide ring at carbon 4″, although in some instances the fatty acids of the SLs may remain in the free acid, open-chain form.

One of the perceived benefits of SLs to the producing organisms is to help access and utilize lipophilic substrates (Ito and Inoue 1982). However, their large production capacity (reportedly as high as 422 g/l when using whey and rapeseed oil as substrates; Daniel et al. 1998) and amphiphilic nature have also increased awareness for their application in some industrial arenas (Solaiman et al. 2004). Acetylated lactones have been used as additives in shampoo, body washes, detergents (Hall et al. 1995; Inoue et al. 1980) and in cosmetic products and have been documented to have bacteriostatic activity (Mager et al. 1987) while the acidic form of SLs has been found to be therapeutically active for skin treatments (Maingault 1999) and as moisturizing agents (Abe et al. 1981). To a certain extent, structural variation (and hence physical properties) can be achieved by changing the lipidic carbon source, which alters the SL fatty acid content.

In this study, SLs were synthesized by *C. bombicola* from C16 (palmitic acid) and C18 (stearic acid, oleic acid, and linoleic acid) fatty acids, their content distribution accurately determined and their surface active properties correlated to their molecular content. In addition, the resulting SLs were blended together in different ratios, providing a simple but effective method to fine-tune SL properties without the need for more costly chemical modifications.

Materials and methods

Materials

All media components were purchased from Sigma-Aldrich Chemical Company with the exception of the yeast extract which was distributed by Fluka Biochemika (Buchs, Switzerland) and all solvents were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI).

Sophorolipid synthesis

Candida bombicola ATCC 22214 inoculum was prepared in Candida Growth Media (CGM), which consisted of the following in g/l: glucose, 100; yeast extract, 10; and urea, 1, according to a previously published method (Ashby et al. 2005). Sophorolipid synthesis was performed in 2 l medium in a 2.5 l bench-top fermenter (Bioflo III Batch/Continuous Bioreactor, New Brunswick, NJ). The basal SL production medium (CGM medium) was made up with the same components and in the same ratios as described above. The pH was brought to pH 6.0 with concentrated HCl and the CGM media was sterilized by autoclaving. After sterilization, the temperature was equilibrated to 26 °C and palmitic acid, stearic acid, oleic acid or linoleic acid was added to the media as the lipid co-substrate at 2% (w/v) resulting in a final pH prior to inoculation of 5.8 ± 0.2 for

each culture. Palmitic acid and stearic acid were added as insoluble solids while oleic acid and linoleic acid were added as non-miscible liquids. A frozen inoculum culture, 50 ml, was thawed and used to inoculate each fermentation. The growth/production conditions were as follows: temperature = 26°C, agitation (impeller speed) = 700 rpm, air-flow = 2 l air/min and no pH control. After 2 days, an additional 5% (w/v) dry glucose and 2% (w/v) fatty acid were added to the fermentations and the fermentations allowed to proceed to 5 days when an additional 0.5% (w/v) fatty acid was added. The fermentations then continued for an additional 2 days (total duration of the fermentation was 7 days).

Sophorolipid isolation and purification

To isolate the SLs, the entire culture (cells and broth) was divided into equal aliquots and lyophilized to dryness ($\sim$2 days). The dried residues were placed into 1 l Erlenmeyer flasks, and each portion extracted with excess ethyl acetate by shaking at 250 rpm, 30°C for 2 days. Periodically throughout the extraction process, 50 ml ethyl acetate were removed and tested for the possibility of preferential solubility of lactone vs. open chain form by TLC (for procedure see below). The extracts were filtered through Whatman No. 2 filter paper, and the remaining solids were washed twice more with ethyl acetate (500 ml each time) to maximize recovery. The combined ethyl acetate fractions containing the SLs were concentrated by evaporation and added to 1 l hexane to precipitate the pure SLs. The pure SLs were recovered from the hexane by filtration and vacuum-dried in a desiccator to obtain a fine white powder, which was then accurately weighed to obtain the product yield.

Sophorolipid analysis

Preferential SL solubility and fractionation in ethyl acetate was elucidated by TLC using silica gel plates (250 μm layer thickness, 17 μm particle size, 60 Å pore size (Sigma-Aldrich) and chloroform/methanol/water (80:20:1, by vol. as solvent). The developing reagent was 5% (w/v) phosphomolybdic acid in ethanol followed by charring.

The SL content was determined as described previously (Nuñez et al. 2001). In short, the SL mixtures were separated by HPLC with a Waters 2690 Separation Module (Waters Company) using a 15 cm × 2.1 mm Symmetry C18 3.5 μm column. A linear gradient elution was used from water/acetonitrile (0.5% formic acid)/acetonitrile (50:10:40, by vol.) and held for 5 min to acetonitrile (0.5% formic acid)/acetonitrile (10:90, by vol.) over 25 min with a total running time of 35 min. The flow rate was 0.25 ml/min. The effluent was connected to a Micromass ZMD mass spectrometer with an APCI probe (Waters) set to the positive mode to scan from *m/z* 200 to *m/z* 1000 at 2 seconds per scan. Corona pin voltage was tuned to 3.8 kV, sample cone 20 V and extraction cone 2 V for detection of fragments and molecular ions ($[M]^+$).

Surface-active property measurement

Oil-water interfacial tension (IFT) was measured on a drop volume Krüss DVT30 tensiometer (Krüss USA, Charlotte NC). The water phase contained 200 mg surfactant/l, 0.01 M Na_2SO_4, 0.77 mM $CaCl_2$, 0.26 mM $MgCl_2$, pH 8.0. Canola (rapeseed) oil was used as the oil phase. An average of three measurements was recorded at 10 μl oil/min. Critical micelle concentration (CMC) and minimum surface tension (min.ST) were determined using a Kibron Delta-8 tensiometer (Kibron Inc., Helsinki, Finland). The sample solution contained 0.01 M Na_2SO_4, 0.77 mM $CaCl_2$, 0.26 mM $MgCl_2$, and various concentrations of surfactant, with pH 8.0. All physical property measurements were carried out at room temperature.

Results and discussion

The relatively large production capability, renewable nature and "ecofriendliness" of sophorolipids (SLs) are helping to boost their industrial significance as additives or substitutes for some petrochemical surfactants in home and personal care products. However, in order to be considered for industrial use, SLs must also be produced inexpensively and their physical properties must be understood and controlled in order to accurately predict the potential of SLs in various applications. Surface tension (ST), critical micelle

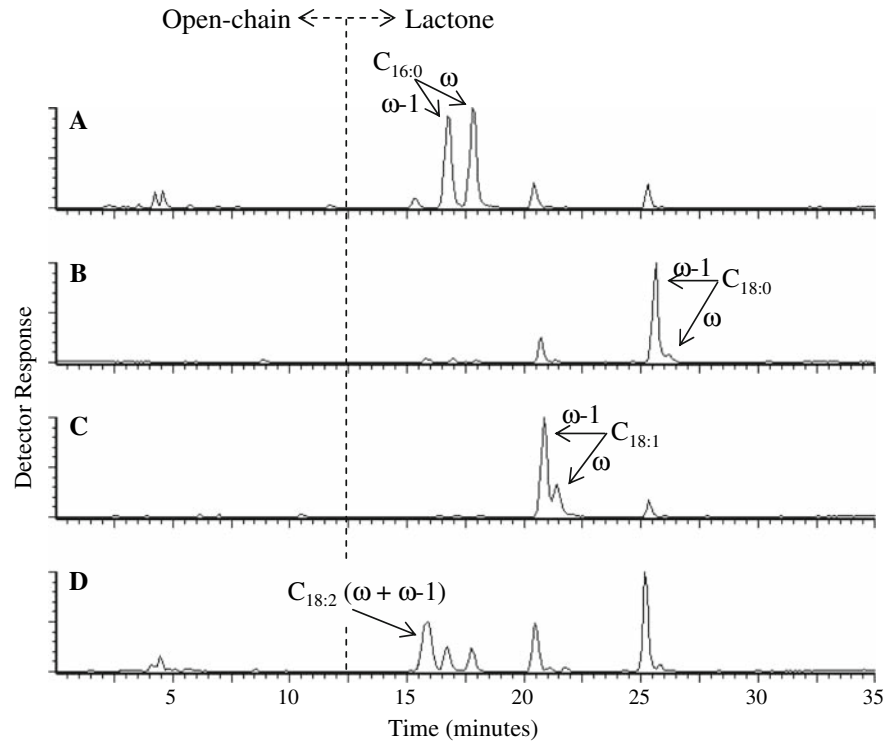
concentration (CMC), interfacial tension (IFT), and water solubility are four properties that are of particular interest for surfactant applications. Ideally, a good surfactant is water-soluble, exhibits a low CMC and results in minimum surface tensions (min.ST) less than 30 mN/m. Each of these properties can be controlled according to the chemical make-up of the surfactant. By growing *C. bombicola* on a number of different mixed substrates (including glucose and 4 different fatty acids), we have produced SLs with varying chemical distributions. The ability to produce different SLs simply by changing the fatty acid provided the prospect of managing physical properties at the fermentation level and, by mixing the SLs in varying ratios, at the application level.

SLs can be biologically produced in relatively large quantities and that the majority of SLs are synthesized in the lactonic form with side chains varying primarily from 16 to 18 carbon atoms. With this in mind, fermentations were conducted in which *C. bombicola* was used to synthesize SLs from palmitic acid (a $C_{16:0}$ fatty acid) and three different C18 fatty acids [including stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$)] in the hope that the majority of the side chains of the resulting SLs would reflect the starting fatty acid substrate. Then, by mixing the various SLs, property control could be achieved without the need for chemical modification, which increases the overall cost of the molecules. Under the fermentation condition employed, *C. bombicola* grew and synthesized SLs in yields of 42 g/l on palmitic acid, 77 g/l on stearic acid, 98 g/l on oleic acid, and 40 g/l on linoleic acid. The increase in yield of SL from stearic acid (SL-s) and SL from oleic acid (SL-o) when compared to the yield of SL from palmitic acid (SL-p) and SL from linoleic acid (SL-l) was due primarily to the enzymatic preference for stearic acid and oleic acid as SL side chain constituents. It is widely recognized that pH affects enzyme efficiency. In all four of the SL fermentations the starting pH values after fatty acid addition were 5.8 ± 0.2 and while the pH was not controlled during the fermentations, it was monitored and within 24 h (post-inoculation) as the cells began to grow, the pH of each fermentation decreased to pH 2.5 ± 0.5 where it remained throughout the balance of the fermentations. In all cases, regardless of the specific fatty acid added, the pH conditions in the fermentations were comparable; therefore, it appears that the difference in SL yield was the result of enzyme-substrate specificity rather than pH difference.

Figure 2 shows the total ion chromatogram (TIC) for each of the 4 SLs produced. In each case the major peaks eluted above 15 min. The SL-p and SL-l (Fig. 2a, d) both showed 5 major peaks while SL-s and SL-o (Fig. 2b, c) only had 3 major peaks each.

The lack of any appreciable peaks between 15 and 20 min in B and C indicated that the peaks that did elute between 15 and 20 min in A and D corresponded to SLs containing palmitic acid and linoleic acid and that the peaks that eluted at 21 min and 25 min corresponded to SLs containing oleic acid and stearic acid, respectively. This was confirmed by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). Figure 3 shows the APCI-MS for each of the base peaks in the TIC.

In each of the mass spectra the peaks showed the same trends regardless of the molecule. For example, each of the mass spectra showed a large base peak corresponding to the protonated molecular ion ($[M]^+$). This peak varied from *m/z* 663 (Fig. 3a) to *m/z* 691 (Fig. 3b), *m/z* 689 (Fig. 3c) and *m/z* 687 (Fig. 3d) depending on the length and degree of unsaturation in the SL side chain. In each case the $[M]^+$ ions sequentially lost a total of 3 water molecules, resulting in ions with *m/z* values that were 18 mass units less than the preceding ions. In addition, each $[M]^+$ lost an acetylated hexose sugar (mass = 204 units), resulting in $[M]^+$-204 values of *m/z* 459, *m/z* 487, *m/z* 485, and *m/z* 483 (Fig. 3a through d), respectively, with an additional loss of 2 water molecules. Lastly, the ions at *m/z* 273, *m/z* 301, *m/z* 299, and *m/z* 297 from Fig. 3a through d, respectively, correspond to the protonated hydroxy fatty acid ($[FA(OH)]^+$) associated with each SL molecule. By using the values associated with each peak it was possible to identify each peak in the TIC and quantitate the absolute distribution of each SL molecule. As hoped, each fatty acid substrate resulted in a large number of SL molecules that contained that particular fatty acid as a side chain constituent. The preference for stearic acid and oleic acid was clarified in that these 2 fatty acids were present in measurable quantities in the SL side chains even when palmitic acid and linoleic acid were used as substrates. These results indicated that *C. bombicola* will enzymatically



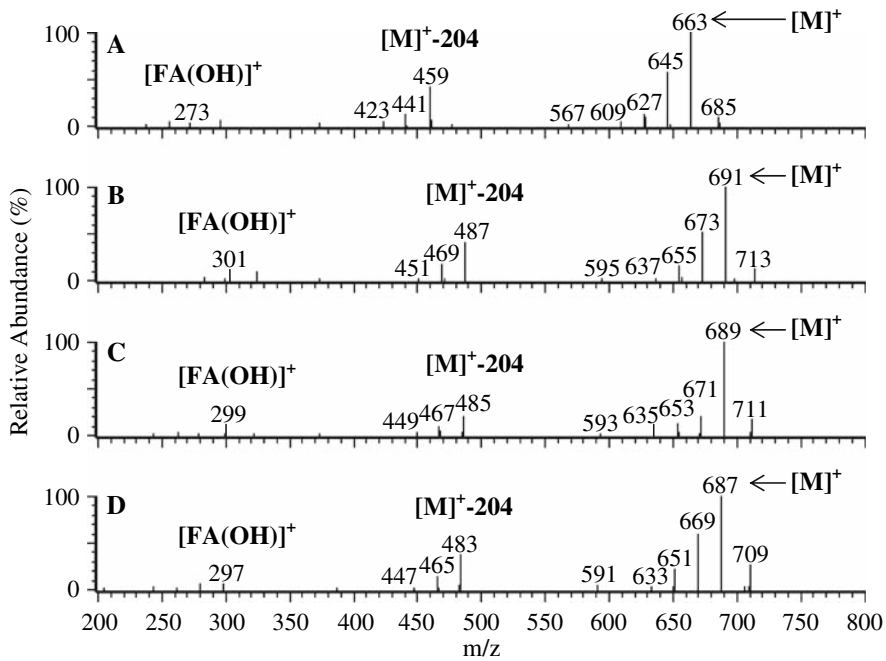


Fig. 2 Total ion chromatographic (TIC) profile of the sophorolipid products produced from glucose and palmitic acid (SL-p; **a**), stearic acid (SL-s; **b**), oleic acid (SL-o; **c**), and linoleic acid (SL-l; **d**)

Fig. 3 Positive atmospheric pressure chemical ionization (APCI)-mass spectra from the base peaks from each TIC chromatogram in Fig. 2. $C_{16:0}$ diacetylated sophorolipid lactone (RT = 16.7 min) from TIC of Fig. 2, $C_{18:0}$ diacetylated sophorolipid lactone (RT = 25.2 min) from TIC of Fig. 2, $C_{18:1}$ diacetylated sophorolipid lactone (RT = 20.7 min) from TIC of Fig. 2c, and $C_{18:2}$ diacetylated sophorolipid lactone (RT = 15.8) from TIC of Fig. 2d. ($[M]^+$ = protonated molecular ion; $[M]^+ - 204$ = protonated molecular ion *-minus-* acetylated hexose sugar (mass 204); $[FA(OH)]^+$ = protonated hydroxy fatty acid)

convert at least some of the substrate fatty acid to stearic acid or oleic acid prior to forming the SL molecule. In contrast, the lack of peaks between 15 and 20 min in the TIC chromatograms for SL-s and SL-o revealed that very little conversion to palmitic acid or linoleic acid occurred prior to SL synthesis, thus proving the enzymatic preference for stearic acid and oleic acid in SL synthesis and further explaining the increased yields of SL-s and SL-o.

The peaks in the TIC (Fig. 2) corresponding to SLs containing palmitic acid, stearic acid and oleic acid all showed a second peak with an identical mass spectrum. From previously reported data (Nuñez et al. 2001) we concluded that of the two peaks, the peak that eluted first corresponded to a structural variation with a glycosidic linkage between the 1′-hydroxy group of the sophorose ring and the $\omega - 1$ carbon of the fatty acid while the later-eluting peak (commonly a minor peak except for SL-p) was linked via a glycosidic linkage from the sugar ring to the ω carbon of the fatty acid. Based on the APCI-MS results, it was determined that in each case, regardless of substrate, greater than 92% of the SL molecules were in the lactone conformation (Table 1).

This was the case regardless of the duration of ethyl acetate extraction. Our protocol included three separate extractions of two days each. These durations were chosen to maximize solubility and hence SL recovery. However, by checking for preferential solubility as a function of time, it was determined that the number of spots that developed on the TLC plates corresponded to the number of compounds present in the TIC of each SL sample (see Fig. 2) which were identified as diacetylated lactones by APCI-MS (see Fig. 3). The absolute distributions of each of the

Table 1 Structural content (open-chain versus lactone) for sophorolipids produced by *C. bombicola* from palmitic (SL-p), stearic (SL-s), oleic (SL-o), linoleic (SL-l) acids as determined by LC/APCI-MS[a]

Sophorolipid sample	Open chain (%)	Lactone (%)
SL-p	8	92
SL-s	1	99
SL-o	<1	100
SL-l	2	98

[a] All sophorolipids were synthesized using glucose as the carbohydrate source (co-substrate)

Table 2 Diacetylated lactone distribution for sophorolipids produced by *C. bombicola* from palmitic (SL-p), stearic (SL-s), oleic (SL-o), linoleic (SL-l) acids and their mixtures according to hydroxy fatty acid content[a]

Sophorolipid sample (mix ratio)	Hydroxy fatty acid content (%)[b]					
	$C_{16:1}$	$C_{17:0}$	$C_{18:2}$	$C_{16:0}$	$C_{18:1}$	$C_{18:0}$
Parental sophorolipids						
SL-p	1	3	–	75 (36)[c]	7 (7)	6 (6)
SL-s	–	–	3	5 (3)	15 (15)	76 (70)
SL-o	–	–	–	–	94 (67)	8 (8)
SL-l	–	–	29	17 (8)	17 (17)	35 (33)
Sophorolipid mixtures						
SL-p/SL-s (50:50)	1	2	1	48 (24)	11 (10)	29 (27)
SL-p/SL-o (50:50)	–	1	–	46 (23)	44 (35)	6 (6)
SL-p/SL-l (50:50)	1	2	9	63 (32)	9 (9)	10 (10)
SL-s/SL-o (50:50)	–	–	2	2 (1)	55 (45)	40 (36)
SL-s/SL-l (50:50)	–	–	11	8 (4)	14 (14)	65 (60)
SL-o/SL-l (50:50)	–	–	9	7 (4)	69 (56)	16 (16)
SL-p/SL-s/SL-o/SL-l (25:25:25:25)	–	–	5	28 (14)	29 (26)	37 (35)

[a] All sophorolipids were synthesized using glucose as the carbohydrate source (co-substrate)

[b] Percentages were determined by peak integration from the TIC chromatograms

[c] All parenthetical values under "Hydroxy Fatty Acid Content" correspond to the % of that sophorolipid that was in the $\omega - 1$ conformation. Those entries without parenthetical values were unresolvable under the LC/APCI-MS method used

SLs including ratios of ω to $\omega - 1$ linkages (where possible) are shown in Table 2.

Of the 4 SLs, only one, SL-p, showed a larger total of ω-linkages than $\omega - 1$-linkages. Specifically, of the 75% of the palmitic acid residues, 39% were linked through the ω position while 36% were linked through the $\omega - 1$ position. In the other cases, the large majority (at least 71%) of the fatty acids were bound through the $\omega - 1$ position (linoleic acid was unresolvable under the LC method used and therefore was reported as combined ω and $\omega - 1$ structures). These results suggested that in order to form the lactone, there must be at least 15 and as many as 17 carbon units between the glycosidic bond and the ester linkage. The large preference for $\omega - 1$ attachment in SL-o and SL-s suggested that 17 carbons are preferred for lactone formation. However, by attaching palmitic acid to the SL at the ω carbon, the chain length is increased by 1 carbon unit (16 carbons between bonds), making the formation of the lactone more energetically favorable than when attached at the $\omega - 1$ carbon (15 carbons between bonds). This was further supported by the existence of SLs with low levels (3%) of $C_{17:0}$ fatty acids in SL-p, while the SL-s, SL-o and SL-l showed no signs of $C_{17:0}$ fatty acid inclusion. Based on these data and the diacetylated lactone distributions (seen in Table 2) it was concluded that the order of preference for the individual fatty acids tested for SL inclusion was oleic acid, stearic acid, palmitic acid, and linoleic acid and that of the C18 fatty acids tested, monounsaturation is preferable to saturation and diunsaturation.

The surface active properties were tested to determine the effects of each SL on the water solubility, min.ST, CMC, and IFT. We found that SL-p and SL-l both had water solubilities greater than 200 mg/l while SL-s and SL-o both had water solubilities less than 200 mg/l. Figure 4 shows the effects of SL concentration on the surface tension of water (Fig. 4a) and the effects of flow rate on the IFT (Fig. 4b).

Each of the 4 parental SLs resulted in comparable min.ST of 35–36 mN/m but the CMC values were noticeably different (Table 3). The SL-p and SL-l both had CMC values greater than 200 mg/l while SL-s and SL-o displayed CMC values of 35 mg/l and 140 mg/l, respectively. The IFT values ranged from 3 mN/m (SL-p) to 7 mN/m (SL-l). In an attempt to control the surface active properties of the SLs,

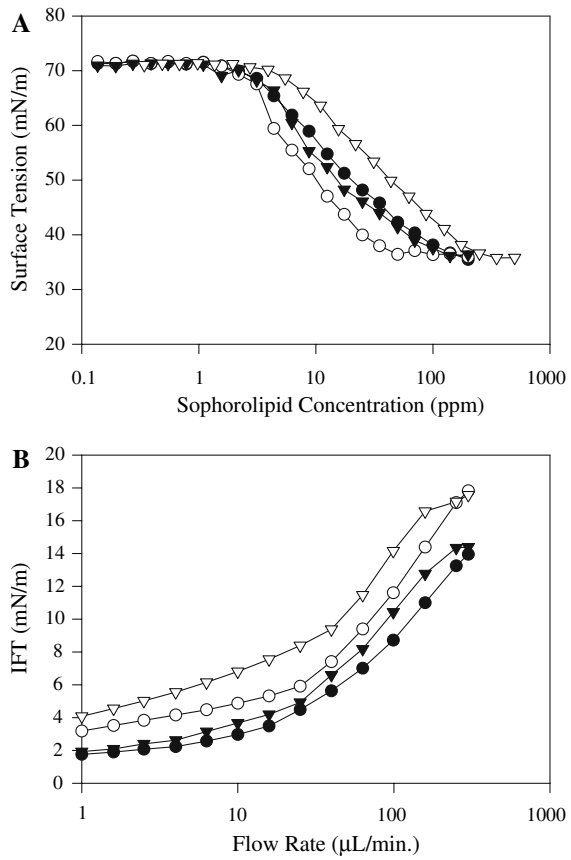


Fig. 4 Effects of sophorolipid concentration and flow rate on surface tension (**a**) and interfacial tension (**b**) in aqueous solution including sophorolipids from glucose and palmitic acid (SL-p; ●), stearic acid (SL-s; ○), oleic acid (SL-o; ▼), and linoleic acid (SL-l; ▽)

mixtures were made at mix ratios of 50:50 and the properties reevaluated. The lactone distributions of each of the mixtures can be seen in Table 2. Upon analysis, the min.ST and the IFT were relatively unchanged, but the CMC values did vary based on the SL mixture. Critical micelle concentrations of between 48 mg/l and 140 mg/l were achieved through mixing. Interestingly, the SL mixtures with the lowest CMC values each had an SL-s component to them. Specifically, SL-s had a CMC value of 35 mg/l; by mixing SL-s with SL-p, SL-o and SL-l in 50:50 ratios the CMC values increased to 63 mg/l, 50 mg/l and 48 mg/l, respectively. Even the mixture containing all 4 parental SLs in a 25:25:25:25 ratio exhibited CMC values (70 mg/l) that were relatively low compared to the other mixtures without an SL-s component. These data show that SL-s was superior

Table 3 Physical properties for sophorolipids produced by *C. bombicola* from palmitic (SL-p), stearic (SL-s), oleic (SL-o), linoleic (SL-l) acids and their mixtures[a]

Sophorolipid sample (mix ratio)	Water solubility (mg/l)	min.ST[b] (mN/m)	CMC[b] (mg/l)	IFT[b] (mN/m)
Parental sophorolipids				
SL-p	>200	35	>200	3
SL-s	<200	35	35	5
SL-o	<200	36	140	4
SL-l	>200	36	250	7
Sophorolipid mixtures				
SL-p/SL-s (50:50)	<250	36	63	4
SL-p/SL-o (50:50)	>200	36	140	3
SL-p/SL-l (50:50)	<200	36	140	4
SL-s/SL-o (50:50)	<200	36	50	4
SL-s/SL-l (50:50)	<200	36	48	5
SL-o/SL-l (50:50)	<200	36	160	5
SL-p/SL-s/SL-o/SL-l (25:25:25:25)	<200	36	70	4

[a] All sophorolipids were synthesized using glucose as the carbohydrate source (co-substrate)

[b] Minimum surface tension (min.ST), critical micelle concentration (CMC), interfacial tension (IFT)

with respect to its effect on surface active properties of water. By using less SL-s, it is possible to achieve equal min.ST and IFT. Property control, along with high yield, would ultimately help in the industrial application of SLs by keeping the cost down. These data also point out the possibility of controlling surface active properties of SLs to some extent by mixing different SLs together to achieve the desired properties. While min.STs were not achieved that would indicate superior surfactant properties for these SLs alone, their properties may be sufficient to permit their increased use as additives for petrochemical surfactants in order to minimize adverse environmental impact in applications where large scale surfactant use is common.

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